QTL Analysis Based on A Rice Short-Wide Grain CSSL-Z414 and Substitution Mapping of qGL11 and qGW5

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Abstract

Background: Most of rice agronomic traits as grain length etc. are complex traits controlled by multiple genes. Chromosome segment substitution lines (CSSLs) are ideal materials for dissecting and studying of these complex traits.

Results: We developed a novel rice short-wide grain CSSL, Z414, deriving from progeny of the recipient parent Xihui 18 (an *indica* restorer line) and the donor parent Huhan 3 (a *japonica* cultivar). Z414 contained 4 substitution segments (average length was 3.04 Mb). Compared with Xihui 18, Z414 displayed seven significantly different traits as grain length, width and weight, chalkiness degree, brown rice rate etc. Then, 8 quantitative trait loci (QTLs) were found responding these difference traits by F_2 population from Xihui 18/Z414. Among them, 6 QTLs (*qPL3*, *qGW5*, *qGL11*, *qRLW5*, *qRLW11*, *qGWT5*) could be verified by novel developed single segment substitution lines (SSSLs, S1-S6). In addition, 4 QTLs (*qGL3*, *qGL5*, *qCD3* and *qCD5*) were novel detected by S1 and S5. Thus, the short–wide grain of Z414 was responded by *qGL11*, *qGL3*, *qGL5*, and *qGW5.* Then, *qGL11* and *qGW5* were delimited within intervals of 0.405 and 1.14 Mb on chromosomes 11 and 5, respectively, by substitution mapping. Again by sequencing, qRT-PCR and cell morphology analysis*, qGW5* should be a novel allele of *GS5* and *qGL11* is novel QTL encoding *CycT1;3*, whose specific function of regulating grain length was still unknown. Finally, pyramid of *qGL3* (a=0.22) and *qGL11* (a=-0.19) displayed *qGL11* epistatic to *qGL3*. In addition, novel S1 and D2 exhibited different grain sizes and lower chalkiness degree. They are potential to be directly used in breeding hybrid rice varieties.

Conclusions: We constructed a novel rice short–wide grain CSSL-Z414 with 4 substitution segments based on the genetic backgrounds of Xihui 18. The broad grain of Z414 was controlled by *qGW5*, which should be a novel allele of *GS5.* The short grain of Z414 was controlled by *qGL11*, *qGL3*, and *qGL5*, and *qGL11* is a novel QTL encoding *CycT1;3*, whose specific function of regulating grain length was still unknown, and *qGL11* is epistatic to *qGL3*. Novel S1 and D2 are potential in hybrid rice varieties.

Background

Rice is a staple food for 3.2 billion people. The food security threat that shook many Asian countries in 2008 still looms, because human is facing the challenge of producing more rice with fewer resources of water, land, and inputs (Xu et al. 2021a). It is necessary to continue to improve rice breeding efficiency. At the beginning of the post-genomic era, a new concept of “breeding by design” was proposed, which aims to control all allelic variation for all genes of crop agronomic importance (Zhang. 2021). The core of rice design breeding is the use of naturally occurring variation (Zhang. 2021). First of all, identification of favorable alleles is principle. Here, a rice short –wide grain chromosome segment substitution line (CSSL) was identified from naturally occurring variation. It is important to detect the alleles for grain size in the CSSL for our carrying out the plan of design breeding.

Grain size and weight are important target traits determining rice yield and quality in rice (Feng et al. 2021). At present, more than 400 QTLs related to grain size have been mapped (Huang et al. 2013). Some of them have been cloned. Several signaling pathways that determine grain size have been identified by these cloned genes, including phytohormones pathway, G protein signal pathway, ubiquitin-proteasome pathway, mitogen-activated protein kinase (MAPK) signal transduction pathway, and transcription factor regulation (Li and Li. 2016). Several phytohormones such as brassinosteroids (BRs) and auxins (IAA) have been suggested to play an important role in rice grain size. In rice, Three QTLs (*GS5*, *qGL3/GL3.1* and *GW5*) for grain size might be involved in BR signaling. *GS5* encodes a putative serine carboxypeptidase, and competitively inhibits the interaction between *OsBAK1-7* and *OsMSBP1* by influencing BR signaling. Higher expression of *GS5* results in wide and heavy grains as a result of increased cell proliferation and expansion in spikelet hulls (Li et al. 2011; Xu et al. 2015a). *GL3.1* suppresses BR signaling by regulating the phosphorylation and stability of *OsGSK3* (Gao et al. 2019). *GW5*, as a novel positive regulator of BR signaling, can repress the kinase activity of *GSK2*, resulting in accumulation of unphosphorylated *OsBZR1* and *DLT* proteins in the nucleus to mediate BR responsive gene expression and growth responses, thus affect the grain width (Liu et al. 2017). Two QTLs (*TGW6* and *BG1*) for rice grain size might participate in IAA signaling. *TGW6* encodes indole-3-acetic acid (IAA)-glucose hydrolase. In sink organs, the Nipponbare *tgw6* allele limits cell number and grain length by controlling IAA supply. Loss of function of the Kasalath allele enhances grain weight and yield significantly (Ishimaru et al. 2013). *BG1* controls rice grain size as a positive regulator of IAA response and transport (Liu et al. 2015a). G protein signaling is involved in regulating grain size in rice. In the pathway, heterotrimeric G protein is usually composed of five subunits. Gα subunits of *RGA1* provides a foundation for grain size expansion, Gβ subunits of *RGB1* is essential for plant survival and growth, Gγ subunits of *GS3* act as a brake in this pathway, *GS3* reduces grain length by competing for binding to *RGB1* to inhibit the downstream signal of *DEP1*/ *GGC2* (Sun et al. 2018). Ubiquitin can directly or indirectly regulate grain size by affecting protein transport, signal transduction and degradation. *GW2*, encoding a RING-type E3 ubiquitin ligase, negatively regulates cell division by targeting its substrate to proteasomes for regulated proteolysis. 1 bp missing in the *GW2* allele from WY3 causes the terminated translation prematurely during the transcription process, which makes the substrates that should be degraded cannot be recognition, and the grain length becomes longer (Song et al. 2007). *LG1* encodes *OsUBP15* that possesses de-ubiquitination activity in vitro, loss-of-function and down-regulated expression of *OsUBP15* produced smaller grains (Shi et al. 2019). MAPK cascades transmit developmental signals to target molecules through sequential phosphorylation (Xu et al. 2015b). *OsMKKK10*, *OsMKK4* and *OsMAPK6* act in the same MAPK pathway to regulate grain size as a positive regulator (Xu et al. 2018). *GSN1* is a negative regulator of the *OsMKKK10-OsMKK4-OsMPK6* cascade, and *gsn1* mutant has larger grain size (Guo et al. 2018). Transcription regulators are considered to be important factors in controlling plant seed size. *GS2* affects grain size by encoding a transcription factor *OsGRF4* (Duan et al. 2015). *GW8* as a SBP-domain positive transcription factor regulates grain width, and can bound directly to the *GW7* promoter and repress its expression (Wang et al. 2015).

Although several genes for rice grain size have been cloned, it is still fragmentary for our understanding of the underlying mechanisms that regulate rice grain size. Thus, it is necessary to identify more QTLs for grain size. Here, we identified a rice short-wide grain chromosome segment substitution line (CSSL) Z414 with 4 substitution segments from *indica* restorer line Xihui 18 as recipient parent and Huhan 3 as donor parent. Chromosome segment substitution lines (CSSLs) have become important materials for QTL mapping (Li et al. 2019). In this study, we will clarify the following theoretical problems: Since Z414 contained 4 substitution segments, how many QTLs will respond the difference traits in Z414 and how are they distributing in these substitution segments? If QTLs controlling the same trait are more than one, will they display independent inheritance or epistatic interaction? Which of these QTLs are reported or novel? Hence, we characterize Z414 systematically and map quantitative trait loci (QTLs) for associated traits by a secondary F_2 population derived from a cross between Xihui18 and Z414. And then validate these QTLs and analyze the inheritance model and pyramid effect of target QTLs using the single-segment substitution lines (SSSLs) and dual-segment substitution lines (DSSLs) developed in the F_3 generation. Finally, we also analyze the candidate genes of major *qGL11* and *qGW5*. The findings will be important for our design breeding plan.

Materials And Methods

Materials

Z414 was developed from Xihui 18 as the recipient parent and Huhan 3 as the donor parent. Xihui 18 was an elite *indica* rice restorer line bred by Southwest University, with the characteristics of good combining ability, large panicle and multiple grains, and narrow-long grain. Huhan 3 as a *japonica* variety had the characteristics of strong stress resistance and short-wide grain. Firstly, 429 simple sequence repeats (SSR) markers covering the whole rice genome were used to screen the polymorphisms between Xihui 18 and Huhan 3. Then, 241 polymorphic markers were selected to develop CSSLs beginning from BC_2F_1, 20 plants for each line in each generation were taken. Until in BC_3F_7, a short-wide grain CSSL-Z414 with 4 substitution segments was identified. The identification of chromosome substitution segments referred to the method described by Ma et al (2019), and the estimated length of chromosome substitution segments was calculated according to the method of Paterson et al (1991).

Material planting

Xihui 18 was crossed with Z414 to get hybrid seeds at the experimental station of Southwest University, Chongqing, China in July of 2018, and the hybrid seeds were planted at Lingshui base of Hainan Province in September of the same year and then the F_1 seeds were harvested. On March 10 of 2019, seeds of Z414, Xihui 18, and the F_2 population of 150 plants were sown at the experimental station of Southwest University. Thirty seedlings of each parent and all F_2 individuals were transplanted to the field on April 20 with the spacing between rows of 26.4 and between hills of 16.5 cm, for 10 plants per row. On March 12 of 2020, eight individuals selected from F_2 population for developing SSSLs and DSSLs, as

well as Z414 and Xihui 18 were planted, for each material, 30 plants were transplanted at the experimental station of Southwest University. On march 10 of 2021, six SSSLs and 2 DSSLs together with Xihui 18 and Z414 were planted in Chongqing, again with 30 individuals transplanted per line. Conventional management practices were applied.

Assessment of agronomic traits and quality parameters

In the maturity period, 10 plants of Xihui 18 and Z414 and 6 SSSLs and 2 DSSLs together with 150 individuals of F_2 were harvested. Eleven yield-related traits were investigated including plant height, panicle number per plant, panicle length per panicle, spikelets number per panicle, grains number per panicle, seed-set rate, grain length, grain width, ratio of length to width, 1000-grain weight, and the yield per plant. The specific method followed Wang et al (2020).

Five quality parameters were analyzed referring to the national standard GB/T5495-2008. Firstly 10 g grains of Xihui 18, Z414,150 F_2 individuals, 6 SSSLs and 2 DSSLs were ground into brown rice. The brown rice was milled into polished rice using the CLS.JNM-1 rice husker. And then brown rice rate and head rice rate were calculated. The chalky rice rate and chalkiness degree were measured using all head rice for each sample by Wanshen SC-E. And the gel consistency was measured refer to the method described in Tang et al (1991).

The mean values of each trait were used for further according analysis.

Scanning electron microscope analysis of glumes in Z414 and Xihui 18

At the completion of the booting stage and before the heading period, the inner and outer epidermal cells of the glume in Xihui18 and Z414 were investigated using a Hitachi SU3500 scanning electron microscope (Hitachi, Tokyo, Japan) with a frozen stage (−40℃) under a low-vacuum environment.

QTL mapping

QTL mapping population was a secondary F_2 population consisted of 150 individuals derived from crosses between Xihui18 and Z414.The improved CTAB method described by McCouch et al(1988) was used to extract DNA from the parents and 150 F_2 individuals. PCR amplification, polyacrylamide gel electrophoresis and rapid silver staining were carried out by the method described by Zhao et al (Zhao et al. 2016). Xihui 18 lanes were scored as “−1”, Z414 lanes were scored as “1”, heterozygous lanes were scored as “0”, and the absence of marker lanes was scored as “.”. Lanes of each marker located on the substitution segment of Z414, together with the phenotypic value of each individual of the F_2 population were used to identify putative QTL using the restricted maximum likelihood (REML) method implemented in the HPMIXED procedure in SAS 9.3 (SAS Institute Inc., Cary, NC, USA). The *P*-value <0.05 was used as the threshold to determine whether a QTL linked to a certain marker on the substitution segment of Z414.

Development of SSSLs and DSSLs, and validation and epistatic interaction analysis of target QTLs

Development of SSSLs and DSSLs

According to QTL mapping in 2019, eight individuals with target segment and less heterozygous markers selected from the F_2 were used to develop SSSL and DSSL by molecular marker-assisted selection (MAS) method. Each was planted as a line (Z728-Z735) in 2020. Then, the leaves of 20 individuals for each line were taken to extract DNA to conduct molecular detection using both the target substitution markers and the residual heterozygous markers by MAS. Development of SSSL and DSSL obeyed the rule that each substitution line carried only the homozygous target substitution segment while the lanes of other markers were same with Xihui 18.

Ten plants of 6 SSSLs, 2 DSSLs and Xihui 18 in each plot were harvested after maturity in August of 2021. All the involved traits were measured again with the same methods in 2019.

Validation of target QTLs by SSSLs

Concerning each $SSSL_i$ (S1-S6), given that the theoretical hypothesis H0: no any QTL was existed on the substitution segment of $SSSL_i$. Then when *P*-value was less than 0.05 by student *t*-test, we denied the hypothesis and considered that a QTL for a certain trait existed in $SSSL_i$. According to the genetic model under certain environment (same year and same experimental field and no replicate plot designed), $P_0 = \mu+\varepsilon$ for Xihui18 and $P_i = \mu+a_i+\varepsilon$ for SSSL carrying a specific QTL, where, P_0 and P_i represented the phenotype value of any plant in plot of Xihui 18 and the $SSSL_i$. μ represented the mean value of Xihui18 population, a_i represented additive effect of the QTL, ε represented random error. We took the half of the difference of phenotype value into account as caused by inheritance. Thus, additive effect of the QTL was calculated as half the difference between the mean phenotypic values of SSSL and Xihui18 (Liang et al, 2021). All calculations were conducted in Microsoft Excel 2016.

Epistatic interaction analysis between target QTLs by DSSLs

Regarding each $DSSL_{ij}$, given that the theoretical hypothesis H0: two loci for a certain trait located in “i” and “j” substitution segment belonged to independent inheritance, showed as “2+0=1+1”. Then when *P*-value was more than 0.05 by comparing (Xihui18 + $DSSL_{ij}$) and ($SSSL_i$+ $SSSL_j$) using student *t*-test, we accepted the hypothesis that two loci belonged to independent inheritance. At this time, the phenotype value of (Xihui18 + $DSSL_{ij}$) was the same with ($SSSL_i$+ $SSSL_j$). In contrast, when *P*-value was less than 0.05 by student *t*-test, we denied the hypothesis and considered that epistatic interaction occurred between the two allelic loci, namely “2+0 ≠1+1”. According to the genetic models, $P_0 = \mu+\varepsilon$ for Xihui18, $P_i = \mu+a_i+\varepsilon$ for $SSSL_i$, $P_j = \mu+a_j+\varepsilon$ for $SSSL_j$ and $P_{ij} = \mu+a_i+a_j+l_{ij}+\varepsilon$ for $DSSL_{ij}$, where P_{ij} represented the phenotype value of any plant in plot of the $DSSL_{ij}$, μ represented the mean value of Xihui18 population, a_i and a_j represented the additive effect of QTL in substitution segment i and j, respectively. l_{ij} represented the a_ia_j epistatic effect between QTLs in substitution segment i and j. We also took the half of the difference of phenotype value into account as caused by inheritance. Thus, the epistatic effects between

non-allele QTLs were estimated as half of the mean phenotypic values of (Xihui18+$DSSL_{ij}$)-($SSSL_i$+$SSSL_j$) (Liang et al. 2021). Finally, we used IBM SPSS Statistics 25.0 to conduct multiple comparison for all SSSLs and DSSLs as well as Xihui18.

Overlapping substitution mapping and candidate gene analysis of *qGL11* and *qGW5*

For *qGW5*, we developed three secondary SSSLs (S3-S5) with overlapping substitution segments in the F_3 population in 2020. For *qGL11*, we constructed 5 SSSLs (S7-S11) with overlapping substitution segments in the F_2 population from Xihui 18 /S6 in 2021. The maximum and estimated substitution length of the according secondary SSSLs were estimated by the marker positions (Paterson et al,1991)). The QTLs were located by substitution mapping (Yang et al. 2021). When GL or GW showed significant difference between secondary SSSL genotype and Xihui 18 genotype, a QTL for GL or GW was detected on the substitution segment of SSSL. When multiple substitution segments in SSSLs with target trait overlapped, the QTL was located in the overlapping region (Yang et al. 2021). The additive effect of the QTL was calculated as half the difference between the mean phenotypic values of SSSL and Xihui18 (Liang et al, 2021). Within the maximum substitution intervals, we predicted the candidate gene information and combined with gene annotations to screen the possible candidate genes of *qGL11* and *qGW5* by the Gramene (http:/www.gramene.org/) and the Rice Annotation Project Data-base (https:/rapdb.dna.affrc.go.jp/) and the China National Rice Database Center (http:/www.ricedata.cn/). The candidate gene sequence, including 3000 bp before the start codon ATG and 1500 bp after the stop codon was downloaded, and the primers were designed on Vector NTI to amplify the target fragments using DNA of Xihui 18 and corresponding SSSL as templates, respectively. The amplicons were sequenced by Tsingke Biological Technology Co., Ltd. (Chongqing, China).

Total RNA extraction and qRT-PCR analysis

Total RNA was extracted from root, stem, leaf, sheath and panicle of Xihui 18 and SSSLs using the RNAprep Pure Plant RNA Purification Kit (Tiangen, Binjing, China) in the booting stage, and reverse transcribed using the GoScript Reverse Transcription System, and then analyzed quantitatively on a Bio-Rad CYF96 using real-time PCR Master Mix (TaKaRa Biotechnology (Dalian, China) Co. Ltd.).

Results

Identification of substitution segments and phenotype analysis of Z414

In the study, 8 polymorphic SSR markers on the substitution segments and 233 polymorphic SSR markers outside the substitution segments of Z414 were used to detect the molecular backgrounds of Z414. The results showed that the substitution segments of 10 plants of Z414 were consistent and no other residual segments from Huhan 3 were detected. Z414 contained 4 substitution segments from Huhan 3, which

were distributed on the chromosome 3, 5 and 11. The total estimated length of substitution segments was 12.17 Mb, and the average length was 3.04 Mb (Fig. 1).

Compared with Xihui 18, Z414 displayed significant increase in grain width (Fig. 2c), 1000-grain weight, brown rice rate and chalkiness degree by 23.5%, 16.4%, 8.8% and 26.1%, respectively (Fig. 2f, h, i, j). While there was significant decrease in panicle length, grain length and ratio of length to width of Z414 (Fig. 2b, c), reducing by 17.5%, 7.7% and 25.1%, respectively (Fig. 2d, e, g). There were no significant differences in the other traits (Fig. 2a), such as plant height, panicle number per plant, spikelet number per panicle, grain number per panicle, seed-set rate, yield per plant, head rice rate, chalky rice rate and gel consistency (no difference data not shown).

Cytological analysis of the glumes in Z414 and Xihui 18

Since the grain length and width of Xihui 18 was different from Z414 (Fig. 3a, d), Scanning electron microscopy was used to analyze the cell morphology of glumes in Xihui 18 and Z414 at the heading stage. The cell width in the inner epidermis of the glumes of Z414 was increased significantly than that of Xihui 18 by 22.23% (Fig. 3c, f, h). The total cell number in the outer epidermis of the glume along the longitudinal axis of Z414 was reduced significantly than that of Xihui 18 by 13.52% (Fig. 3b, e, i). The cell length in the inner epidermis of the glumes of Z414 exhibited no significant difference compared with that of Xihui 18 (Fig. 3c, f, g). The results indicated that the short-wide grain of Z414 was mainly caused by decrease of glume cell number and increase of glume cell width.

Identification of QTL using the secondary F_2 population from Xihui18/Z414

A total of 8 QTLs were detected on 3 substitution segments of Z414. They explained the phenotypic variation from 3.86–50.39% (Table 1). The allele *qGW5* from Huhan 3 increased the grain width of Z414 by 0.12 mm, explaining 50.39% of the phenotypic variation. The additive effect of *qGWT5* from Huhan 3 increased 1000-grain weight of Z414 by 0.63 g. Furthermore, *qGW5*, *qRLW5* and *qGWT5* all linked to the same marker RM5874. The allele *qGL11* from Huhan 3 reduced the grain length of Z414 by 0.08 mm, explaining 9.96% of the variation in grain length. Similarly, *qGL11, qRLW11*, *qGWT11* and *qBRR11* all linked to the same marker RM1812. However, the additive effects of *qGL11*, *qRLW11*and *qGWT11* decreased the values of corresponding traits, while *qBRR11* increased the value (Fig. 1, Table 1). In addition, the additive effect of *qPL3* reduced panicle length of Z414 by 0.58 cm per panicle (Table 1).

Table 1
QTL for agronomic and quality traits identified in substitution segments of Z414

Trait	QTL	Chr.	Linked marker	Additive effect	R^2 (%)	P-value
Panicle length (cm)	*qPL3*	3	RM3766	-0.58	9.02	0.0295
Grain width(mm)	*qGW5*	5	RM5874	0.12	50.39	0.0013
Grain length (mm)	*qGL11*	11	RM1812	-0.08	9.96	0.0138
Ratio of length to width	*qRLW5*	5	RM5874	-0.10	38.38	0.0038
Ratio of length to width	*qRLW11*	11	RM1812	-0.04	7.15	0.0069
1000-grain weight (g)	*qGWT5*	5	RM5874	0.63	10.47	0.0450
1000-grain weight (g)	*qGWT11*	11	RM1812	-0.37	3.86	0.0457
Brown rice rate (%)	*qBRR11*	11	RM1812	1.19	5.92	0.0309

Verification and pyramid of QTLs using the developed SSSLs and DSSLs

On the basis of primary QTL mapping, 6 single segment substitution lines (SSSLs, S1-S6) and 2 dual-segment substitution lines (DSSLs, D1, D2) were developed in F_3 by MAS method. Among them, S3, S4, and S5 belonged to SSSLs with overlapping substitution segments (Fig. 4a).

6 QTLs (*qPL3*, *qGW5*, *qGL11*, *qRLW5*, *qRLW11*, *qGWT5*) could be verified by SSSLs (Fig. 4a-f), which indicated that these QTLs could be inherited stably. 2 QTLs (*qGWT11* and *qBRR11*) were not validated by S6, suggesting that the expression of some minor QTLs might be easily influenced by the environment, whose contribution rates to phenotypic variation were only 3.86% for *qGWT11* and 5.92% for *qBRR11*. In addition, 4 QTLs (*qGL3*, *qGL5, qCD3* and *qCD5*) for grain length and chalkiness degree were detected by S1 and S5(Fig. 4c, g), which were not detected in the secondary F_2 population (Table1). The results showed that SSSLs had a higher efficiency of QTL detection.

The Panicle length (25.49 cm) of S1 carrying *qPL3* (a=-0.77) was significantly shorter than that (27.03cm) of Xihui 18, while those of S2-S6 without QTL for PL displayed no significant differences with that of Xihui 18 (Fig. 4a, b). Grain length (9.66 and 9.55 mm) of S5 carrying *qGL5* (a=-0.13) and S6 with *qGL11* (a=-0.19) were significantly shorter than that (9.92 mm) of Xihui18, while grain length (10.35 mm) of S1 carrying q*GL3* (a= 0.22) was significantly longer than that (9.92 mm) of Xihui 18, and S2-S4 without QTL for GL exhibited no significant differences with Xihui 18 (Fig. 4a, c). Grain width (3.59 and 3.52 mm) of S4 and S5 harboring *qGW5* (a=0.23 and a=0.20) were significantly wider than that (3.12 mm) of Xihui 18, while those of S1, S2, S3 and S6 without QTL for GW showed no significant differences with Xihui 18. The results indicated that *qGW5* was located in the same substitution interval of RM405–RM5874-RM3322–RM3328 of chromosome 5 by theory of substitution mapping (Fig. 4a, d). 1000-grain weight (34.40 and 33.43 g) of S4 and S5 containing *qGWT5* (a=1.83 and a=1.34) were significantly larger than that (30.75 g) of Xihui 18, and the other SSSLs (S1, S2, S3, S6) without QTL for GWT had no

significant differences with Xihui 18. And *qGWT5* was in the same substitution interval of chromosome 5 with *qGW5* (Fig. 4a, e). Ratio of length to width (2.75, 2.75 and 2.99) of S4, S5 carrying *qRLW*5 (both a=-0.22) and S6 harboring *qRLW11* (a=-0.10) were significantly less than that (3.19) of Xihui 18, and those of S1- S3 without QTL for the trait displayed no significant differences with that of Xihui 18 (Fig. 4a, f). Chalkiness degree (19.76%) of S1 harboring q*CD3* (a=-0.59) was significantly lower than that of Xihui18 (20.93%), while chalkiness degree (24.02%) of S5 carrying *qCD5* (a=1.54) was significantly higher than that (20.93%) of Xihui 18, the other SSSLs (S2, S3, S4 and S6) without QTL for CD displayed no significant differences with Xihui 18 (Fig. 4a, g).

Pyramid of *qGL3* (a= 0.22) and *qGL11* (a=-0.19) yielded an epistatic effect of -0.31, which resulted in reduction 0.28 mm of the grain length in D2. The result suggested that pyramid of *qGL3* and *qGL11* resulted in shorter grains than S6 (containing *qGL11*) (Fig. 4c), indicating that *qGL11* displayed epistatic to *qGL3*. However, *qGL3* (a= 0.22) and a substitution locus without GL on chromosome 3 in D1 belonged to independent inheritance. The grain length (10.25 mm) of D1 exhibited no significant difference with that (10.35 mm) of S1, while significantly longer than that of Xihui18 and S2 (Fig. 4a, c). Pyramid two substitution loci without QTL for 1000-grain weight on chromosomes 3 and 11 in D2 produced an epistatic effect of -2.94, resulting in decrease 2.94 g of 1000-grain weight genetically in D2. Thus, 1000-grain weight (28.99 g) of D2 displayed significantly lower than that (32.65, 31.98, and 30.75 g) of S1, S6 and Xihui18 (Fig. 4a, e). As for the other QTLs in D1 and D2, they all belonged to independent inheritance (Fig. 4b, d, f, g).

Substitution mapping and candidate gene analysis of qGL11 **and** qGW5

candidate gene analysis of qGL11

According to the above results, we firstly dissected *qGL11* into S6 whose estimated and maximum substitution length was 1.42 Mb and 1.66 Mb, respectively (Fig. 5a). In order to fine mapping of *qGL11*, we developed 5 novel secondary SSSLs (S7-S11) by a cross of Xihui 18 and S6. By theory of substitution mapping, *qGL11* was finally delimited into 405 Kb of the estimated substitution interval (Fig. 5a). There were 44 genes in total were found in the estimated substitution interval, only 18 genes with specific functional description except the others annotated as 16 expressed protein, 6 transposon protein and 2 retrotransposon protein and 2 carrier & putative protein. Then, we found only *LOC_Os11g05850* (*CycT1;3*) might be the candidate gene of *qGL11* according to the possible signaling pathway regulating grain size (Li and Li. 2016), By DNA sequencing between Xihui 18 and S6, there were 6 SNP differences and a 25-base insertion in the 5'UTR and 1 SNP difference in the 3'UTR, and 1 SNP difference in the CDS which did not cause amino acid change (Fig. 5b). Furthermore, the protein structure displayed no difference between S6 and Xihui18 (Fig. 5c). Especially, expression levels of *LOC_Os11g05850* was significantly higher in sheath and panicle in S6 than in Xihui18 (Fig. 5d). Thus, *LOC_Os11g05850* (*CycT1;3*) should be the candidate gene for *qGL11*.

Substitution mapping and candidate gene analysis of qGW5

Using substitution mapping of S3, S4 and S5, *qGW5* was delimited into 1.135 Mb of the estimated substitution length and 1.33 Mb of the maximum substitution interval between RM405 and RM17984 (Fig. 6a). Within the interval, we found 6 genes involved in the reported signaling pathway for grain size, including *LOC_Os05g06270* (*APIP6*, RING E3 Ubiquitin Ligase), *LOC_Os05g06280* (*SRS3*, small and round seed 3), *LOC_Os05g06660* (*GS5*, regulator of grain size), *LOC_Os05g06320* (*OsERS2*, ethylene receptor), *LOC_Os05g06670* (*OsGA2ox1*, gibberellin 2-oxidase gene) and *LOC_Os05g07720* (*OsTAR1*, IAA biosynthesis gene). By DNA sequencing of these genes, only *LOC_Os05g06660* (*GS5*) and *LOC_Os05g07720* (*OsTAR1*) were found existing differences between Xihui 18 and S5. Concerning *LOC_Os05g06660* (*GS5*), as a cloned major QTL for grain size, $(GGC)_7$ repeat after the 90th base of the CDS were found in S5, while only $(GGC)_5$ repeat in Xihui 18, and the GGC encodes Glycine (Fig. 6b). Moreover, the protein structure also displayed some differences between S5 and Xihui18 (Fig. 6c). For *LOC_Os05g07720* (*OsTAR1*), as an IAA biosynthesis gene, there were 4 SNP differences in the CDS between Xihui18 and S5, of which 3 caused amino acid mutations and 1 nonsense mutations (Fig. 6d). Furthermore, qRT-PCR analysis showed that expression levels of the *LOC_Os05g06660* (*GS5*) were significantly higher in stem, leaf, sheath and panicle in S5 than in Xihui18 (Fig. 6e), while no significant expression differences of the *LOC_Os05g07720* (*OsTAR1*) were found in all organs between Xihui 18 and S5 (Fig. 6f). Thus, *LOC_Os05g06660* (*GS5*) should be a different allele of *qGW5* and *LOC_Os05g07720* (*OsTAR1*) were potential one for *qGW5*.

Discussion

We construct a novel short-wide CSSL-Z414 and novel series of secondary SSSLs for different grain size, which are valuable for both genetic study for grain quality and hybrid rice breeding

Restorer line plays an important role in heterosis utilization, and restorer lines are very important for breeding hybrid rice varieties. Xihui 18 was an elite *indica* rice restorer line bred by Southwest University. It had many advantages as good combining ability, large panicle and multiple grains per panicle and narrow-long grain. In this study, we constructed a novel short-wide grain CSSL-Z414 in the genetic backgrounds of Xihui 18. Z414 carried 4 chromosome substitution segments from donor Huhan 3 and displayed seven traits differences, including grain length, grain width, ratio of length to width, 1000-grain weight, brown rice rate, chalkiness degree and panicle length. Again, none of their substitution segments contained the fertility restoration genes *Rf-1*, *Rf2*, *Rf3* and *Rf4* (Akagi et al. 2004; Itabashi et al. 2011; Cai et al. 2013), so Z414 is restorative. Z414 carried 8 QTLs for seven different traits and displayed short-wide and larger grain, shorter panicle, and higher brown rice rate and chalkiness degree when compared with Xihui18. Thus, Z414 did not suit to be directly acted as rice restorer line due to its high chalkiness degree. While it is an ideal material for genetic study due to its near isogenic habit. Luckily, by genetic dissection, we obtained 6 novel single segment substitution lines (S1-S6) and 2 dual-segment substitution lines (D1-D2) harboring target QTLs. Intriguingly, compared with Xihui 18, S1 carried *qGL3* and *qCD3* and exhibited both long grain and lower chalkiness degree. D2 harbored *qGL3, qGL11* and *qCD3* where *qGL11* was epistatic to *qGL3* and thus displayed shorter grain and lower chalkiness degree.

S4 contained *qGW5*, *qRLW5* and *qGWT5* and exhibited long-wide large grain and same chalkiness degree with xihui18. S6 carried *qGL11* and *qRLW11* and showed short-narrow grain and same chalkiness degree with xihui18. Lower chalkiness degree is a very important factor affecting rice grain quality (Yang et al. 2021). Again, different grain sizes are import to meet the requirement of various people's preferences (Feng et al. 2021; Liang et al. 2021). Thus, these four SSSLs are potential to be directly used as novel rice restorer lines to breed novel hybrid rice varieties. As for S5 with short-wide grain (*qGL5*, *qGW5*, *qGWT5*, *qRLW5*) and high chalkiness degree (*qCD5*), it can be used in researching the formation mechanism of these traits. Zhang (2021) argued that SSSLs is helpful for rapid screening of traits hidden in genomes of different donors, make a large amount of previously unexplored genetic variation rapidly available to plant breeders and geneticists, and make the genetic variation directly usable for breeding. SSSLs represents a new resource that could greatly enrich conventional rice breeding (Zhang. 2021). Obviously the novel SSSLs is an unusual gene pool for genetic research for grain quality and rice breeding. They will play important part in alleles discovery and implement the strategy for research on rice breeding by design.

qGW5 **should be a different novel allele of** GS5 **and** qGL11, qRLW11, qCD3 **and** qBRR11 **should be novel QTLs compared with the reported genes**

To explore the problems proposed in introduction, we developed the secondary F_2 segregation population from Xihui 18/Z414 and 6 SSSLs (S1-S6) and 2 DSSLs (D1 and D2), Finally a total of 12 QTLs were found to be responsible for seven difference traits of Z414. Based on the analysis of these QTLs, the short grain of Z414 was controlled by *qGL11*, *qGL3* and *qGL5*, which was dissected into S6, S1 and S5, respectively. The wide grain of Z414 was responded by *qGW5*, which was then validated by S4 and S5. Large grain of Z414 was in charge of *qGWT5* (verified by S4 and S5) and *qGWT11* (verified by S6). The short panicle of Z414 was harbored by *qPL3* (validated by S1). Higher brown rice rate of Z414 was explained by *qBRR1*. The *qCD5* and *qCD3* (detected by S5 and S1) answered for the higher chalkiness degree of Z414.

Compared with the reported genes, *OsAPC6* and *OspPLAIIIa* can be as the candidate genes for *qPL3* according to its physical distance and biological function. *OsAPC6* interferes with the gibberellin signal pathway and leads to cell reduction (Awasthi et al. 2012). *OspPLAIIIa* encodes glycoprotein-related phospholipase A and reduces panicle length (Liu et al. 2015b). According to the results in the study, *qGW5* for grain width, *qGL5* for grain length, *qRLW5* for ratio of length to width and *qGWT5* for 1000-grain weight were within the interval of RM405 to RM17984 of chromosome 5. At the substitution interval, *GS5* and *OsTAR1* were potential candidate genes of these QTLs. *GS5* is a positive regulator of grain size such that grain width and weight are correlated with its expression level. Polymorphisms of *GS5* in the promoter region are responsible for the variation in grain size. Xu et al (2015b) showed that two SNPs between the wide-grain allele *GS5-1* and the narrow-grain allele *GS5-2* in the upstream region of the gene that were responsible for the differential expression in developing young panicles. By DNA sequencing of *GS5*, we found that two GGC repeat encoding Glycine were added in CDS sequence of S5 compared with that of Xihui18, and the protein structure also existed some differences in S5 compared

with Xihui18. While there were no differences found in the promoter region between Xihui18 and S5. Again, qRT-PCR analysis showed that the expression level of *GS5* was significantly higher in stem, leaf, sheath and panicle in S5 than in Xihui18. Thus, *qGW5* should be a different allele of *GS5*, which is important for studying biodiversity of grain size. *OsTAR1* encoding tryptophan aminotransferase, a IAA biosynthesis gene, regulate the production of IAA in the developing rice grain together with *OsYUC9* and *OsYUC11* (Abu-Zaitoon et al, 2012). And *OsYUC11*-mediated auxin biosynthesis is essential for endosperm development in rice (Xu et al, 2021b). By DNA sequencing, there was 4 SNP differences in the CDS between Xihui18 and S5. While the expression of *OsTAR1* displayed no significant differences between Xihui 18 and S5. Thus, *OsTAR1* also can act as the potential candidate gene of *qGW5*. Of course, whether *GS5* and *OsTAR1* are target genes, the genetic complementary experiments of two genes are necessary and they are ongoing. Although *GS5* and *OsTAR1* have been cloned, we have found the different alleles from Huhan 3 and developed the S5 based on the genetic backgrounds of Xihui 18, which can be directly used in our design breeding plan. *qGL3* was in the similar region with *PGL1*, *OsLG3*, *TUD1* and *OsOFP19*. *PGL1* encodes an atypical basic helix-loop-helix protein (bHLH) that does not bind DNA. Its overexpression can increase grain length (Heang et al. 2012). *OsLG3* positively regulates rice grain length and has no effect on grain quality (Yu et al. 2017). *TUD1* encodes an E3 ubiquitin ligase of the U-box family, which participates in the brassinolide response and interacts with the heterotrimeric G protein subunit *D1* to regulate brassinolide-mediated rice growth (Hu et al. 2013). *OsOFP19*, *OSH1* and *DLT* might form a functional complex that regulates cell proliferation and cell growth (Yang et al. 2018). *qCD5* was in the similar region with *Chalk5. Chalk5* encodes a vacuolar H+-translocating pyrophosphatase (V-PPase), which increases the chalkiness of the endosperm by disturbing the PH homeostasis of the endomembrane trafficking system in developing seeds (Li et al. 2014). Although some of these genes have been cloned, when compared with their identification in mutant, they are more usable in breeding practice by identification in SSSLs. Consequently, these alleles are still important for both biodiversity research and pyramid breeding based on genetic background of Xihui 18. In addition, *qGL11*, *qRLW11*, *qCD3* and *qBRR11* have not been previously reported, our knowledge. They can be further used for fine mapping, cloning and functional analysis to explore the genetic mechanisms of these quality trait.

qGL11 **is a novel QTL encoding** CycT1;3 **whose function in regulating grain length was still unknown**

Elucidation of the molecular mechanism underlying grain size is important for rice design breeding. By substitution mapping of *qGL11* using 6 SSSLs (S6 –S11) with overlapping substitution segments each other, *qGL11* was finally delimited within 405 Kb of estimated substitution interval on chromosome 11. At this interval, there were 44 genes in total, in which 18 genes have specific functional description except 26 genes for expressed protein, transposon protein, retrotransposon protein and carrier & putative protein. Considering genes associated with grain size that have been cloned, the majority involved in phytohormones pathway, G protein signal pathway, ubiquitin-proteasome pathway, Mitogen-activated protein kinase (MAPK) signal transduction pathway, and transcription factor regulation (Li et al, 2016). Among the 18 candidate genes, only *CycT1;3* might be the candidate gene for *qGL11*. By DNA sequencing and qRT-PCR analysis between Xihui 18 and S6, there were differences in both DNA

sequences and gene expression levels in sheath and panicle for *CycT1;3* between Xihui 18 and S6. Thus, *CycT1;3* should be the candidate gene for *qGL11. CycT1;3* encodes cyclin protein, involving in biological progress of the cell Mitosis cycle. Intriguingly, cytological analysis showed that the shorter grain of Z414 depended on the decrease of cell number of glume rather than cell length. The results suggest that *qGL11* is related to cell mitosis cycle. There were many studies on cell cycle regulation in yeast and animals, but few studies in plants. Cell proliferation in plants is mainly controlled by a super-family of cyclin-dependent kinases (CDKs). There are more reports of A, B, D and E-type family cyclins in plants (Nieduszynski et al. 2002), but still few reports on T-type family cyclins. Therefore, T-type cyclin in plant is worthy to further study. Qi et al. (2012) found that *GL3.1* encoding *OsPPKL1* can directly use Cyclin-T1;3 as a substrate to phosphorylate Cyclin-T1;3, which makes Cyclin-T1; 3 down-regulated in rice and resulting in shorter grains. However, the molecular mechanism of how *CycT1;3* affects grain development remains unknown. In this study, although we detected *qGL3* in S1 and found that *qGL11* was epistatic to *qGL3*, however, *OsPPKL1* was not in the institution interval of S1. Thus, *qGL11* is a novel QTL. On all accounts, these results are important for the in-depth study of *qGL11*.

Conclusions

We constructed a novel rice short-wide grain CSSL-Z414 carrying 4 substitution segments based on the genetic backgrounds of *indica* restorer line Xihui18. Z414 displayed seven different traits from Xihui 18, including grain length, grain width, ratio of length to width, 1000-grain weight, brown rice rate, chalkiness degree and panicle length. In total, 12 QTLs responded seven different traits of Z414, and they were dissected into 6 novel SSSLs (S1-S6) and 2 novel DSSLs (D1 and D2). The short grain of Z414 was controlled by *qGL11*, *qGL3* and *qGL5.* By Cytological analysis, DNA sequencing and qRT-PCR analysis, *qGL11* should be *CycT1;3*, whose specific function regulating grain length was still unknown and is a novel QTL. The grain width of Z414 was controlled by *qGW5*, and should be a different novel allele of *GS5*. In particular, S1 carried *qGL3* and *qCD3* and exhibited both long grain and lower chalkiness degree. D2 harbored *qGL3, qGL11* and *qCD3* where *qGL11* was epistatic to *qGL3* and thus displayed shorter grain and lower chalkiness degree. They are potential to be directly used as novel rice restorer lines to breed novel hybrid rice varieties.

Abbreviations

QTL: Quantitative trait loci; CSSLs: Chromosome segment substitution lines; SSSL: Single-segment substitution lines; DSSL: Dual-segment substitution lines; SSR: Simple sequence repeat; MAS: Marker-assisted selection; PL: panicle length; GL: Grain length; GW: Grain width; RLW: Grain length-to-width ratio; GWT: 1000-grain weight; BRR: Brown rice rate ; CD: Chalkiness degree; SNP: Single nucleotide polymorphism; UTR: Untranslated region; CDS: Coding DNA sequence

Declarations

Acknowledgments

Professor Shizhong Xu (University of California, Riverside, USA) wrote the stem program for QTL mapping.

Authors' Contribution

FMZ designed the experiment and proposed the structure and content. JL, HXY, GYX, KLD, JJY, SQX, KZ, QLZ, RXL, MML and YHL performed experiment. FMZ, JL and GHH analyzed the data. ZLY was responsible for field management. JL and FMZ wrote the paper. All authors read and approved the final version.

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Availability of Data and Material

The datasets supporting the conclusions of this article are included within the article.

Ethics Approval and Consent to Participate

This study complied with the ethical standards of China, where this research work was conducted.

Consent for Publication

All authors provide their consent for publication.

Competing Interests

The authors declare that they have no conflict of interest.

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Figures

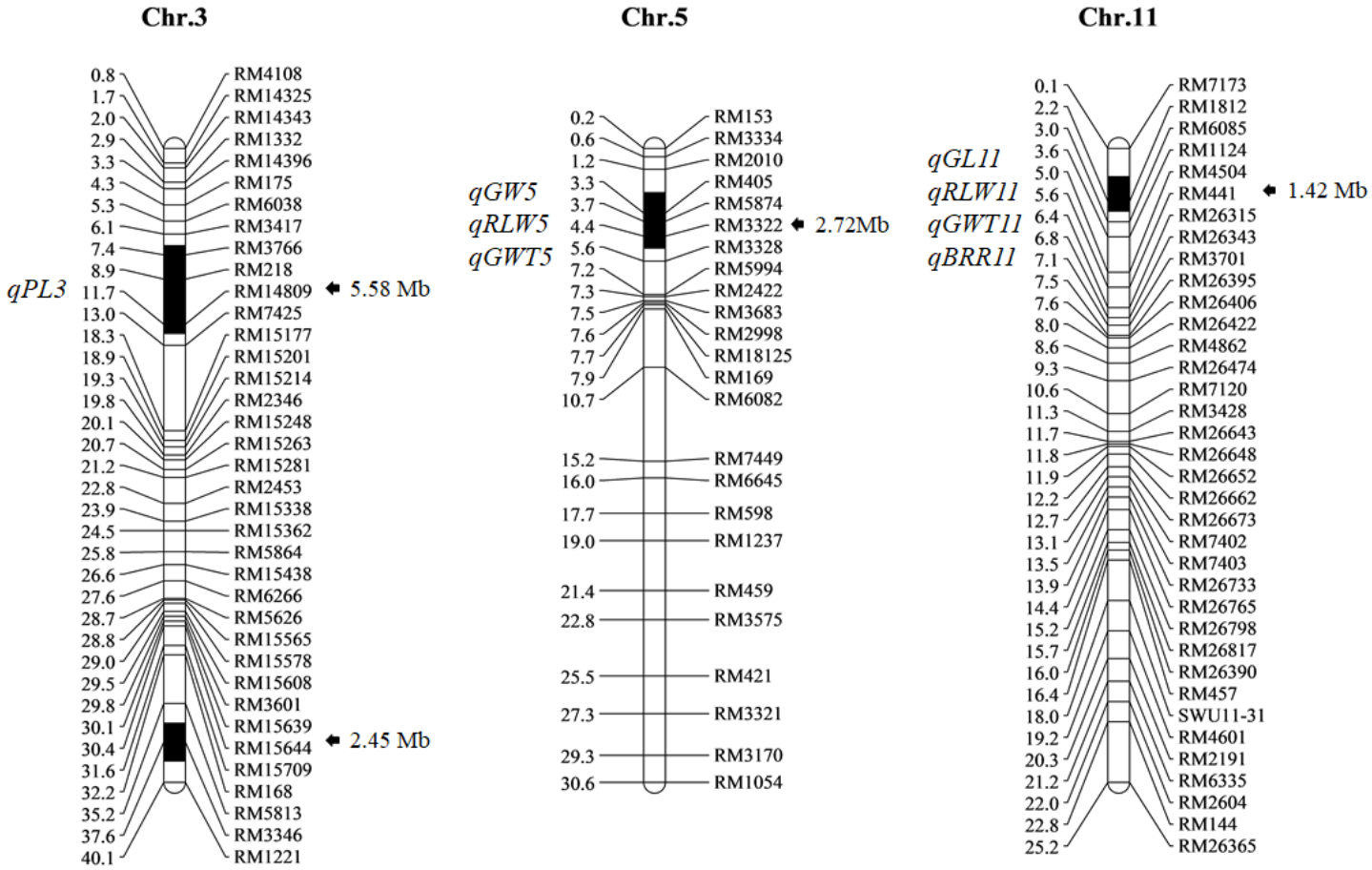


Figure 1

Chromosome substitution segments of Z414. Physical distances (Mb) and mapped QTL are marked at left , markers and substitution segment length are displayed at the right. Black section on each chromosome are substitution segments. PL, Panicle length; GW, Grain width; GL, Grain length; RLW, ratio of length to width; GWT, 1000-Grain weight; BRR, Brown rice rate.

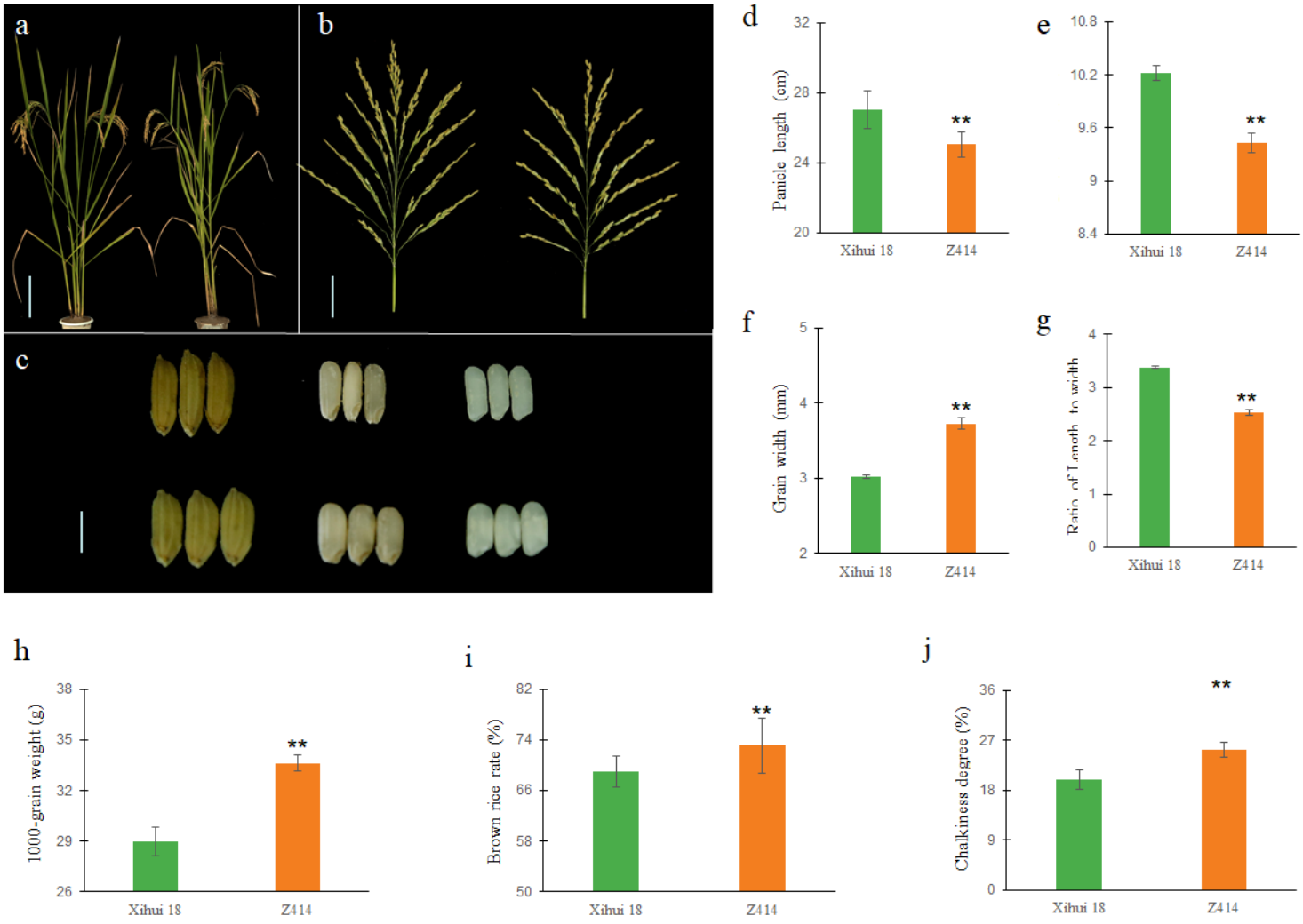


Figure 2

Phenotype of Xihui 18 and Z414. a Plant type of Xihui 18 and Z414. b Panicle of Xihui 18 and Z414. c Grain, brown rice and polished rice of Xihui 18 and Z414. Bars in a represented 20 cm, in b represented 5 cm and in c represented 5mm. d-j, Statistical analysis of seven different traits between Z414 and Xihui 18. * and ** indicated difference at 0.05 and 0.01 level.

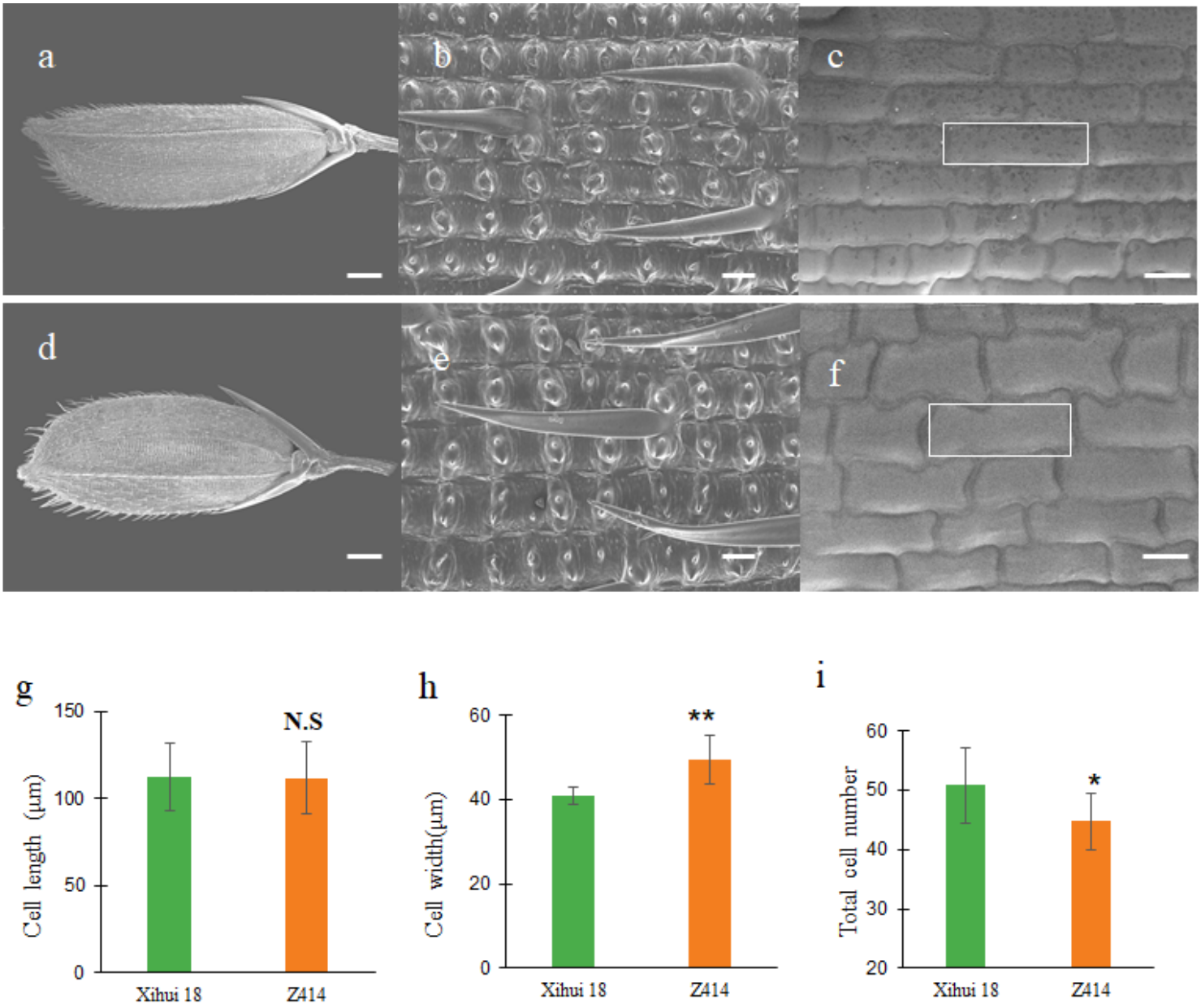


Figure 3

Scanning electron microscopy observation and analysis of glume in Xihui 18 and Z414. a-f Scanning electron microscope of the lemma (a, d), outer epidermis (b, e), and inner epidermis (c, f) of the glume of Xihui 18(a-c) and Z414(d-f). g, h and i represent cell length, width and total number of cells in outer epidermis of lemma at 200x magnification, respectively. ** and *respectively indicates difference at 0.01 and 0.05 level between Xihui 18 and Z414. Bars in a and d represented 10 mm, in b and e represented 500 μm, in c and f represented 500 μm.

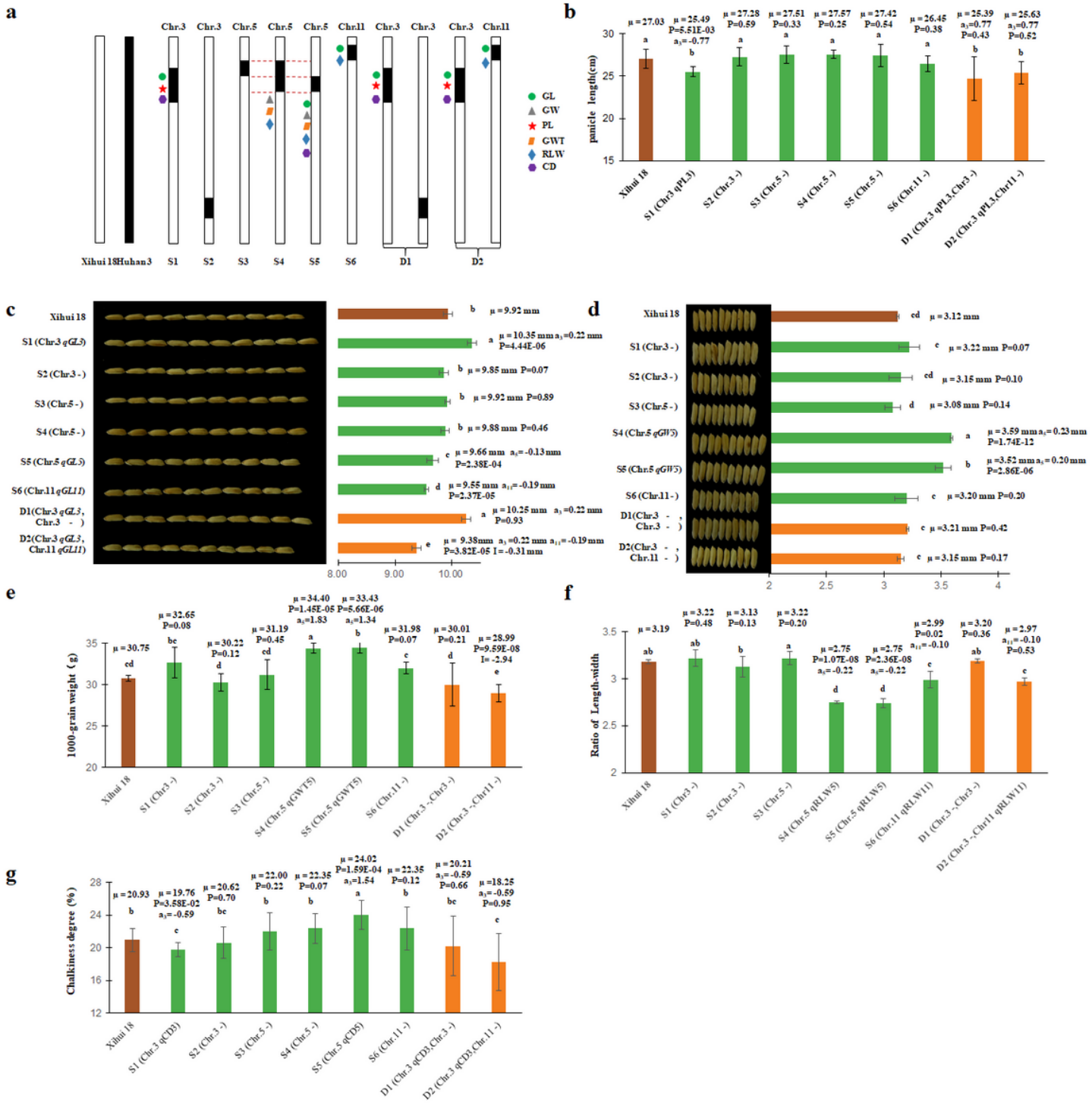
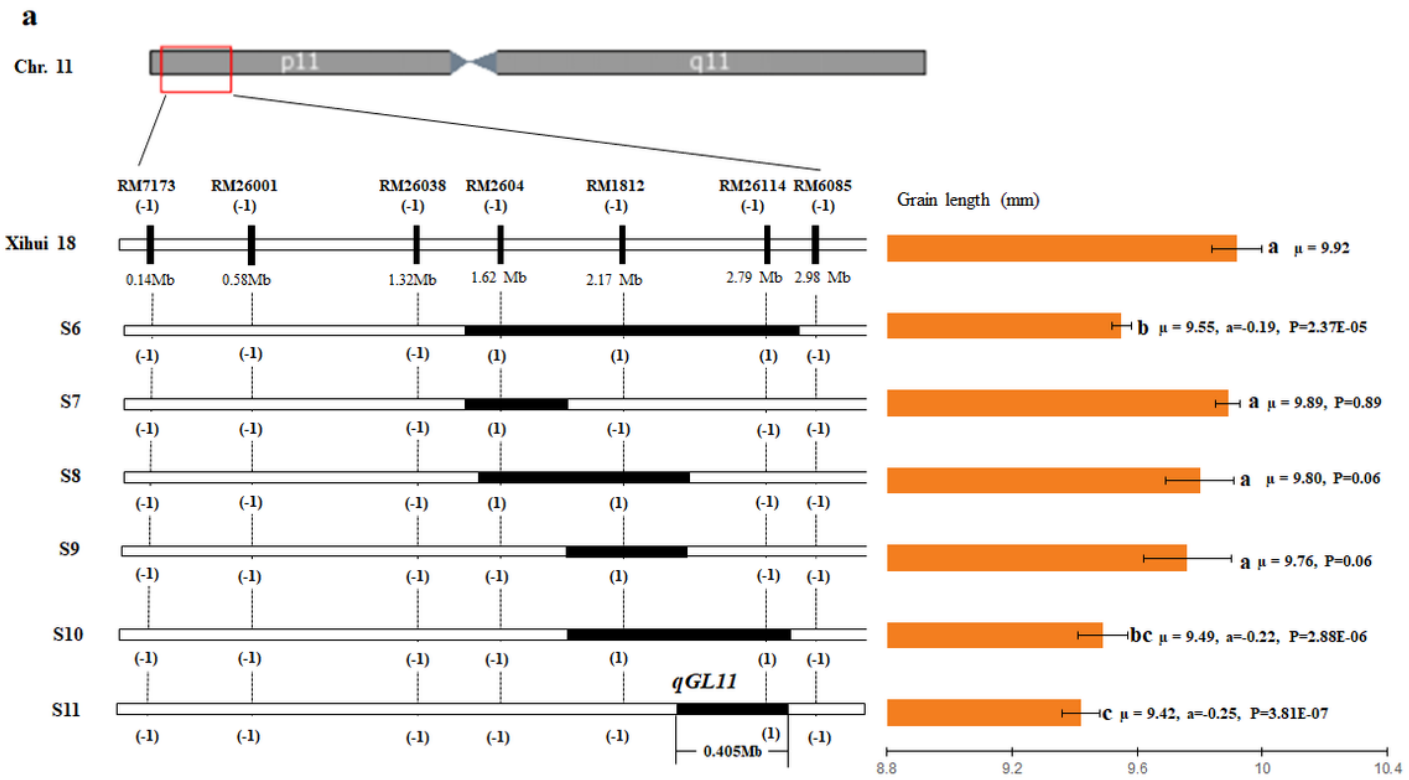
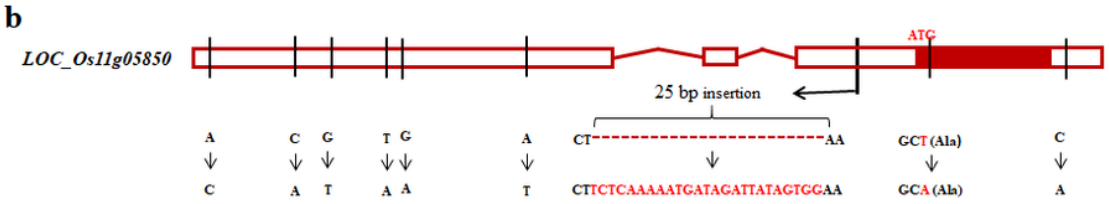


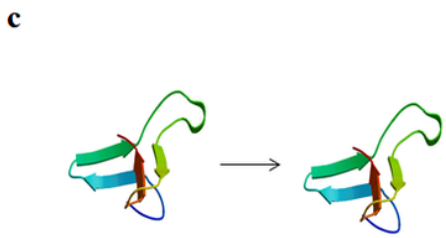
Figure 4

Additive and epistatic effect of QTLs for related traits in SSSL, DSSL. a Schematic diagram of substitution segment and QTL located on them of S1-S4, and D1 and D2; b-f Parameters of QTLs in different SSSLs and DSSLs for panicle length (b), Grain length(c), Grain width (d), 1000-grain weight (e), ratio of grain length to width(f) and Chalkiness degree (g). Different lower-case letters indicate a significant difference ($P < 0.05$) as determined by Duncan's multiple comparasion. μ: mean value of according trait; ai: additive effect for each QTL controlling according trait. I: epistatic effect between

QTLs. P: P-value in SSSL indicated between SSSL and Xihui18 for t-test; P-value in DSSL indicated between (DSSLij+ Xihui18) and (SSSLi+ SSSLj) for t-test. S1:(Chr.3 RM3417–RM3766-RM14809–RM7425); S2(Chr.3 RM5813–RM3346–RM1221); S3(Chr.5 RM2010–RM405–RM5874); S4(Chr.5 RM2010–RM405-RM5874-RM3322–RM3328); S5(Chr.5 RM405–RM5874-RM3322–RM3328); S6(Chr.11 RM26038–RM26045-RM1812-RM26114–RM6085); D1(Chr.3 RM3417–RM3766-RM14809–RM7425, Chr.3 RM5813–RM3346–RM1221); D2(Chr.3 RM3417–RM3766-RM14809–RM7425, Chr.11 RM7173–RM1812–RM6085); The internal markers connected with hyphens indicated the substitution segment from donor, whereas the markers at each end of the substitution segment linked with '–' indicated whether segment recombination might occur.







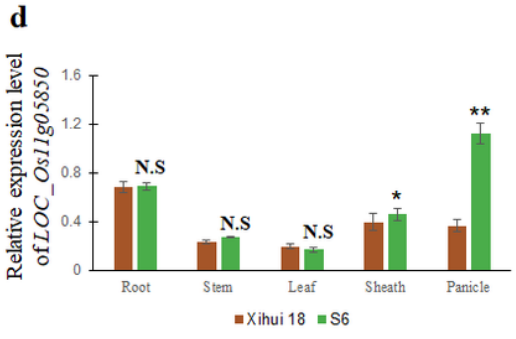


Figure 5

Substitution mapping, sequence analysis and relative expression level of qGL11 between Xihui 18 and S6. a Substitution mapping of qGL11. Black regions indicate the estimated length of substitution segment. b The DNA sequence of CycT1;3 in S6 compared with Xihui 18. In candidate gene sequence, the red box represents the coding sequences region, the white box represents 5'UTR and 3'UTR, the solid red line represents introns, the black line in the gene sequence presents the mutation site, and arrow represents sequence change from Xihui18 to S6. c Protein structure of CycT1;3 predicted by SWISS-MODEL. d Relative expression level of candidate genes CycT1;3 in root, stem, leaf, sheath and panicle between Xihui 18 and S6.

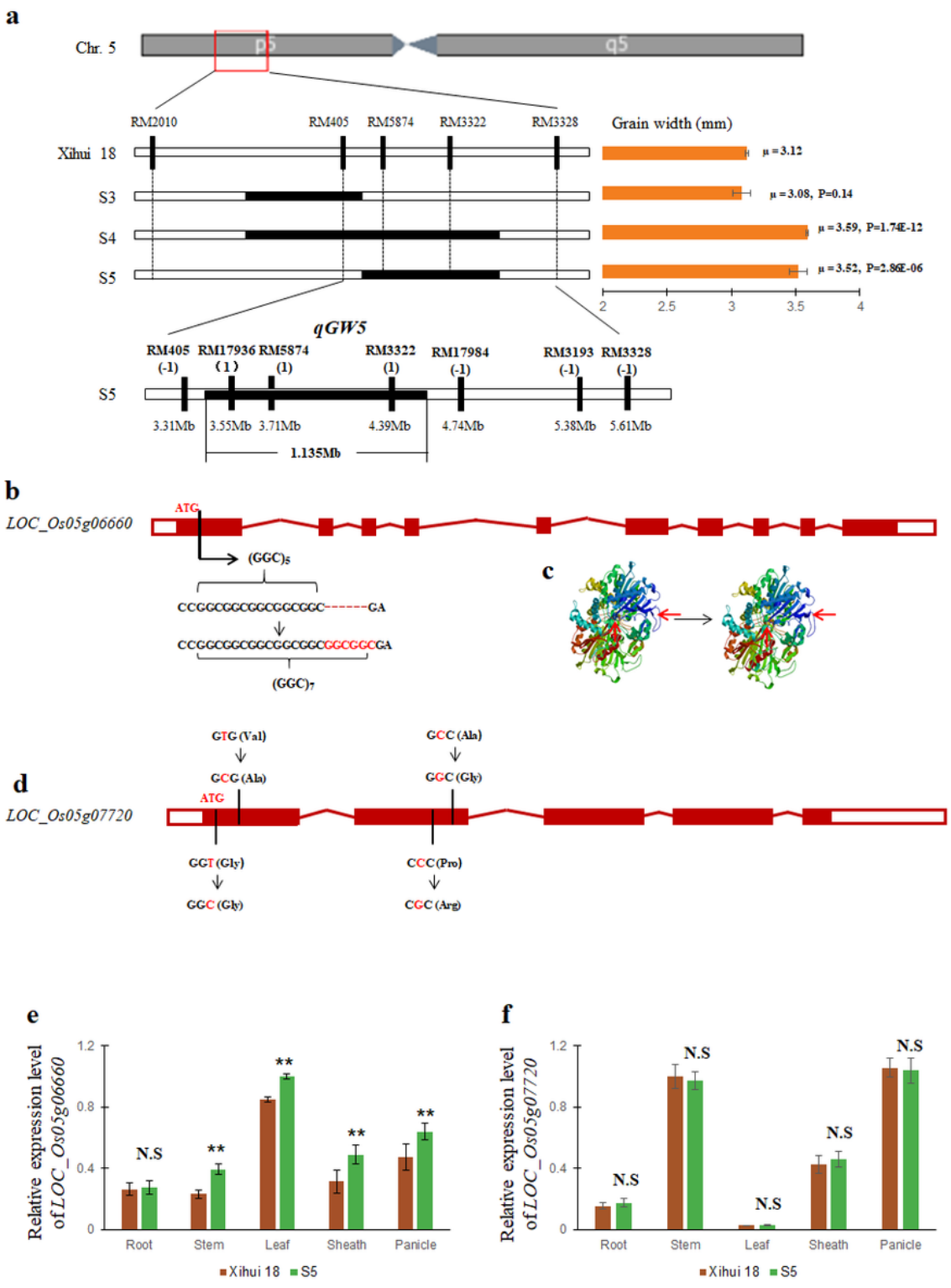


Figure 6

Substitution mapping, sequence analysis and relative expression level of candidate genes for qGW5 between Xihui 18 and S5. a QTL mapping of qGW5. Black regions indicate the estimated length of substitution segment. b The DNA sequence of GS5. c Protein structure of GS5 predicted by SWISS-MODEL.d The DNA sequence of OsTAR1 in S5 compared with Xihui 18. In candidate gene sequence, the red box represents the coding sequences region, the white box represents 5'UTR and 3'UTR, the solid red

line represents introns, the black line in the gene sequence presents the mutation site, and arrow represents sequence change from Xihui18 to S5. e-f, Relative expression level of candidate genes GS5 (e) and OsTAR1 (f) in root, stem, leaf, sheath and panicle between Xihui 18 and S5.